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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/245, A61K 39/102 — A1

(11) International Publication Number: WO 95/25742

(43) International Publication Date: 28 September 1995 (28.09.95)

(21) International Application Number: PCT/IB95/00185

(22) International Filing Date: 20 March 1995 (20.03.95)

(30) Priority Data:
08/216,202 22 March 1994 (22.03.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/216,202 (CIP)
Filed on 22 March 1994 (22.03.94)

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(81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FR; GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: *PASTEURELLACEAE* ANTIGENS AND RELATED VACCINES

(57) Abstract

Antigens of the *Pasteurella, Actinobacillus* and *Haemophilus* species of bacteria capable of being up-regulated during infection in a host animal and in minimal medium formulations which provide protection against infections caused by these species are disclosed. Vaccine compositions containing antigens of the *Pasteurella, Actinobacillus* and *Haemophilus* species of bacteria are also provided along with methods of immunizing animals against infections by these species.

PASTEURELLACEAE ANTIGENS AND RELATED VACCINES

Background of the Invention

Animal vaccines designed to protect against pneumonias caused by *Pasteurellaceae* are generally produced from inactivated whole bacteria or extracts of bacterial cultures. The protective potential of potassium thiocyanate extracts of culture-grown *Pasteurella multocida* have been investigated in mice, chickens, cattle and rabbits. These extracts contained protein, hyaluronic acid, lipopolysaccharide, DNA and RNA, making interpretation of the protective component difficult. Although some cross-protection has been observed, protection was mainly against homologous challenge.

The immunogenic outer membrane proteins expressed by a rabbit isolate of *Pasteurella multocida* grown in culture have also been investigated. The major antibody response appeared to be directed against outer membrane polypeptides having molecular masses of 27 kD, 37.5 kD, 49.5 kD, 58.7 kD and 64.4 kD (Lu et al. (1988) *Infect Immun* **56**:1532-1537). Further work demonstrated that a monoclonal antibody specific for the 37.5 kD protein could passively protect mice and rabbits from challenge, if the isolate used for challenge expressed the antigenic determinant recognized by the monoclonal antibody. However, not all isolates tested expressed the antigenic determinant (Lu et al. (1991) *Infect Immun* **59**:172-180).

Most investigations that concern the cross-protective capacity of *Pasteurella multocida* Type A have used serotypes and isolates that infect poultry. Cross-protective antiserum made in turkeys by inoculating inactivated *in vivo* grown bacteria was used for passive immunization, and results showed this antiserum passively protected young turkeys against heterologous challenge (Rimler RB (1987) *Avian Diseases* **31**:884-887). In an attempt to determine the nature of these cross-protection factors in *Pasteurella multocida*, investigators have shown that

Salmonella typhi (Brown, R.F. and Stocker B.A.D. (1987) *Infect. Immun.* **55**:892-898), *Bacillus anthracis* (Ivanovics et al. (1968) *J. Gen. Microbiol.* **53**:147-162), *Escherichia coli* (Kwaga et al. (1994) *Infect. Immun.* **62**:3766-3772), *Pasteurella multocida* (Homchampa et al. (1992) *Molec. Microbiol.* **6**:3585-3593) and *Yersinia enterocolitica* (O'Gaora et al. (1990) *Microb. Pathogenesis* **9**:105-116). All of these reports suggest that mammalian hosts stringently limit the availability of essential nutrients to bacteria. These results also suggest that bacteria must activate numerous biosynthetic pathways to replicate inside a host and cause a disease. *In vivo* expression technology (IVET), a methodology which selects for bacterial genes that are specifically induced in host tissues, has provided evidence of a nutritionally-exacting environment in a host (Mahan et al. (1993) *Science* **259**:686-688; Mahan et al. (1995) *Proc. Natl. Acad. Sci. USA* **92**:669-673). IVET studies have demonstrated that the *Salmonella typhimurium carAB* and *pheST* genes are specifically induced *in vivo*. Expression of the *carAB* operon results in the increased biosynthesis of arginine and pyrimidines. Induction of the *pheST* operon (which encodes two subunits of phenylalanyl-tRNA synthetase) is believed to be a response to the depletion of a charged tRNA, indicating starvation for the aromatic amino acid phenylalanine.

The *in vivo* activation of microbial biosynthetic pathways provides essential nutrients to bacteria which they are unable to acquire from the host. However, essential mineral requirements cannot be produced biosynthetically and therefore must be obtained from the host. Among these minerals are calcium, magnesium, iron, zinc, copper, manganese and cobalt. The inability to biosynthesize these mineral requirements puts bacteria into a severe nutritional crisis. Metal ion transport has been best studied in iron acquisition. Since bacteria are unable to biosynthesize their own iron, iron restriction places bacteria into a

homologous challenge in turkeys, but did not protect against heterologous challenge.

Antigens from the *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* isolated directly from the pleural cavities of infected swine or from a minimal medium formulation have now been identified. These antigens are proteins which are up-regulated during infection in a host animal and are not observed during culture in standard, enriched media. It has now been found that these antigens are up-regulated in minimal medium formulations. However, these antigens are absent or only weakly expressed during *in vitro* cultivation in standard, enriched media. An immune response to these newly identified antigens invokes protection against heterologous challenge. Therefore, these antigens are useful in the production of an effective vaccine providing cross-protection between multiple isolates of the same species.

Summary of the Invention

An object of the present invention is to provide antigens of the *Pasteurella*, *Actinobacillus* and *Haemophilus* species of bacteria capable of being up-regulated during infection in a host animal and in minimal medium formulations which provide protection against infections caused by these species.

Another object of the present invention is to provide vaccines comprising antigens of the *Pasteurella*, *Actinobacillus* and *Haemophilus* species of bacteria capable of being up-regulated during infection in a host animal and in minimal medium formulations which provide protection against infections caused by these species.

Yet another object of the present invention is to provide a method of immunizing healthy animals against infections caused by *Pasteurella*, *Actinobacillus* and *Haemophilus* species of bacteria which comprises administering to a healthy animal an effective amount of a

collected 18 hours following secondary exposure to isolate 16926.

Detailed Description of the Invention

The *Pasteurellaceae* family of bacteria contains species of the genera *Pasteurella*, *Actinobacillus*, and *Haemophilus*. Recent work on the phylogeny of the *Pasteurellaceae* family confirmed the grouping of these three genera into this family (Dewhirst et al. (1992) *J. Bacteriol.* **174**:2002-2013). The various species within the *Pasteurellaceae* family fall into four large clusters, each cluster containing species of three different genera. Examples of species within this family include, but are not limited to, the animal pathogens *P. multocida*, *A. pleuropneumoniae*, *P. haemolytica*, *H. somnus*, and *A. suis*.

Pasteurellaceae infections in animals result in symptoms similar to those resulting from virulent septic pneumonia. Death is generally due to endotoxic shock and respiratory failure. High mortality rates can occur with the acute form of these infections, however, subacute and chronic forms which result in pleuritis are more common. Treatment of field infections is difficult and often unsuccessful due to widespread antibiotic resistance. Therefore, it is preferred to prevent the infection in animals through use of a vaccine. There has been difficulty, however, in achieving a vaccine which will provide protection against different isolates of a species of bacteria within the *Pasteurellaceae* family.

Cross-protection against different isolates of a species of bacteria within the *Pasteurellaceae* family seems to be dependent upon the ability of the host to mount an immune response against bacterial proteins exclusively expressed under the influence of microenvironmental conditions encountered during infection. Most vaccines designed to protect swine against pneumonias caused by these bacteria are prepared from inactivated whole bacteria.

and other minerals which provide an excellent nitrogen source and general nutritional supplement so that little metabolic demand is placed on the bacteria. In contrast, culture media used in the present invention were designed to supply the bacteria with the minimum level of essential nutrients necessary to support growth, thus mimicking the environment encountered when bacteria invade the host organism. Components of the minimal media used in the present invention comprise basal salts (elemental requirements), carbon sources, special nutritional requirements of the *Pasteurellaceae*, and nonessential optimizing supplements. Examples of basal salts include, but are not limited to, potassium phosphate, potassium sulfate, magnesium chloride, ammonium chloride, calcium chloride and sodium chloride. Examples of elemental requirements include, but are not limited to, potassium, sulfur, phosphorus, sodium, chloride, and calcium. Examples of carbon sources include, but are not limited to, glycerol and lactic acid. Glucose, galactose, fructose, mannose, sucrose, mannitol, and sorbitol can also be utilized by the *Pasteurellaceae*. However, because of the fermentative type of metabolism of these organisms, acid can be produced during catabolism of sugars resulting in a lower yield of bacterial cells in the culture. Thus, use of the non-fermentable carbohydrates glycerol and lactic acid, which do not lead to acid accumulation in cultures, is preferred. In addition, since members of the *Pasteurellaceae* are not prototrophic in that they are unable to grow in a mineral salts medium with a single carbon source, special nutritional additives are required. For example, these species require organic nitrogen sources and may require several amino acids, B vitamins, β-nicotinamide, adenine nucleotides, or protoporphyrin and its conjugates. To satisfy these requirements, the minimal medium may comprise arginine, aspartic acid, cystine, glutamic acid, glycine, leucine, lysine, methionine, serine, tyrosine, inosine,

Table 1

Metals analysis of bacterial growth media

Compound	MM#1	MM#2	MM#3	Complete HP	Detection Limit
Calcium	5.92 mg/L	7.40 mg/L	7.32 mg/L	15.2 mg/L	0.01
Cobalt	n.d. mg/L	n.d. mg/L	n.d. mg/L	0.02 mg/L	0.01
Copper	n.d. mg/L	n.d. mg/L	n.d. mg/L	n.d. mg/L	0.01
Iron	0.09 mg/L	n.d. mg/L	n.d. mg/L	0.46 mg/L	0.05
Magnesium	43.0 mg/L	41/2 mg/L	39.6 mg/L	46.0 mg/L	0.01
Manganese	n.d. mg/L	n.d. mg/L	n.d. mg/L	0.01 mg/L	0.01
Zinc	n.d. mg/L	0.02 mg/L	0.03 mg/L	1.15 mg/L	0.01

complete Heamophilus Pleuropneumoniae (HP) medium containing supplements. The inoculate is incubated at 37°C for several hours, preferably in a shaking incubator. The bacteria are centrifuged at 10,000 x g to remove culture medium and resuspended in sterile PBS (10 mM phosphate, 0.87% NaCl, pH 7.2) prior to use. At least eleven antigens were identified from the Western blot analysis of *in vivo* grown bacteria that are absent from *Pasteurella multocida* grown *in vitro* using in a standard, enriched media. Western blot analysis of bacteria grown *in vitro* in a minimal medium formulation demonstrated that the antigenic profile of bacterial proteins produced in this minimal medium formulation was identical to the antigenic profile produced in a host animal infected by the bacteria. The corresponding protein bands had molecular weights of approximately 115 kD, 109 kD, 96 kD, 89 kD, 79 kD, 62 kD, 56 kD, 53 kD, 45 kD, 34 kD and 29 kD.

The corresponding protein bands for each antigen are then excised from the gel, re-isolated by gel-electrophoresis and transferred onto sequence membranes for N'-terminal amino acid sequencing. The N'-terminal amino acid sequence of a 34 kD antigen is as follows:

Ala Thr Val Tyr Asn Gln Asp Gly Thr Lys Val Asp Val Asn Gly Ser Val Arg Leu Leu Leu Lys Gly Glu Lys Asp Pro Arg Arg Asp Leu Met Met Asn Gly (SEQ ID NO: 1)

The N'-terminal amino acid sequence of 29 kD antigen is as follows:

Ala Asp Tyr Asp Leu Lys Phe Gly Met Val Ala Gly Pro Ser Ala Asn Asn Val Lys Ala Val Glu Phe Ile Ala (SEQ ID NO: 2)

The N'-terminal amino acid sequence of a second 29 kD antigen is as follows:

Lys Phe Lys Val Gln Ile Ala XXX XXX XXX XXX Gln Asp Ile Asn Gln Tyr Tyr Ala Gly Asp Ala Ala Phe Val (SEQ ID NO: 3)

The ability of these antigens to invoke a protective immune response against *Pasteurellaceae* was verified in passive transfer experiments. Antibodies to the bacteria were

a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 96 kilodaltons. In yet another embodiment, a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 89 kilodaltons. In yet another embodiment, a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 79 kilodaltons. In yet another embodiment, a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 62 kilodaltons. In yet another embodiment, a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 56 kilodaltons. In yet another embodiment, a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 53 kilodaltons. In yet another embodiment, a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 45 kilodaltons. In a preferred embodiment, a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 29 kilodaltons and an N'-terminal amino acid sequence comprising SEQ ID NO: 2. In a second preferred embodiment, a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 29 kilodaltons and an N'-terminal amino acid sequence comprising SEQ ID NO: 3. It is also preferred that a *Pasteurella multocida* antigen has a molecular weight, as determined by gel electrophoresis, of approximately 34 kilodaltons and an N'-terminal amino acid sequence comprising SEQ ID NO: 1.

Antigens up-regulated during infection in a host animal and in a minimal media formulation but not in bacteria grown *in vitro* in a standard, enriched media were also identified for isolates of *Actinobacillus pleuropneumoniae*. *A. pleuropneumoniae* is member of the *Pasteurellaceae* family which exists in the most distinct phylogenetic cluster from

intranasally or by suppository at doses ranging from approximately 1 to 100 μg/dose.

In another embodiment, the antigens of the present invention produced *in vivo* or in bacteria grown *in vitro* in a minimal media formulation, recombinantly or via genetic manipulation or under specialized culture conditions can be added to whole culture grown bacteria to produce an effective vaccine. Addition of these antigens to the culture grown bacteria increase the efficacy of the resulting vaccine.

This invention is further illustrated by the following nonlimiting examples.

EXAMPLES

Example 1: Bacterial isolates and growth conditions

Pasteurella multocida isolates 8261 and 16926 were field isolates received from the Iowa State Veterinary Diagnostic laboratory. Both isolates were serotype 3A. For conventional *in vitro* growth, bacteria were inoculated into Heamophilus Pleuropneumoniae (HP) medium (Gibco, Grand Island, NY) containing supplements, and incubated for 6 hours at 37°C in a shaking incubator. The bacteria were centrifuged at 10,000 x g to remove culture medium and resuspended in sterile PBS (10 mM phosphate, 0.87% NaCl, pH 7.2). For *in vivo* growth, 1 ml of cultured bacteria at a concentration of 2 x 10^8 CFU/ml were administered to pigs by transthoracic injection into the diaphragmatic lobe. Pigs were euthanized 16 hours later and *in vivo*-grown bacteria were recovered from the pleural fluids. The pleural fluids were centrifuged at 250 x g to remove large cellular debris, and then *in vivo*-grown bacteria were recovered by centrifugation at 10,000 x g for 40 minutes at 4°C. The bacterial pellet was washed three times with sterile PBS by centrifugation as above. Bacterial pellets were resuspended in PBS and stored at -70°C.

(3×10^9 CFU/ml) were pelleted by centrifugation at 11,000 x g for 40 minutes at 4°C (Beckman microfuge, Beckman Instruments, Palo Alto, CA). The supernatant was removed and 0.1 ml of antiserum added. The mixture was incubated overnight at 4°C while being gently agitated. Following incubation, the adsorbed material was centrifuged at 11,000 x g for 40 minutes and the supernatant was removed and added into a fresh bacteria pellet. The final supernatant was collected and stored at -20°C for Western blot analysis.

For detergent solubilization, the culture grown bacteria was solubilized in a solution containing 0.062 M Tris, 0.069 M SDS and 1.09 M glycerol, pH 7.0. The antigen preparation was then boiled for 10 minutes at 100°C to solubilize the bacteria. After the solubilized antigen had cooled, it was mixed with an equal volume of antibody and incubated overnight at 4°C. The mixture was centrifuged at 20,000 x g for 40 minutes to pellet the precipitated antibody-antigen complexes. The final supernatant was collected and stored at -20°C.

Example 4: Purification of antibody preparations using ammonium sulfate precipitation

Antibody preparations used for the passive immunity study in mice were partially purified using ammonium sulfate. Serum samples were diluted 1:3 in sterile PBS. A solution of saturated ammonium sulfate was diluted to 90% of saturation and then added drop-wise to the diluted serum until a volume equivalent to the diluted serum was added, resulting in a 45% ammonium sulfate precipitation of the antibody. This solution was incubated on ice for 1 hour. The mixture was centrifuged at 10,000 x g for 40 minutes at 4°C to pellet the ammonium sulfate precipitate. The supernatant was discarded and the pellet was resuspended to the initial serum volume using sterile PBS. The ammonium sulfate precipitation was repeated to further purify the antibody. Following resuspension of the antibody, the

34 kD and 29 kD were identified from *in vivo* grown bacteria that were absent from or poorly expressed by culture grown PmA.

Using non-adsorbed sera, the differences in band profiles could not be distinguished between the *in vivo* and culture grown antigens. This indicates that the majority of the antibody response mounted against an infection with *Pasteurella multocida* is specific for antigens that are expressed either when the bacteria are cultured *in vitro* or when the bacteria are recovered from their natural host. In contrast, the majority of antibodies that are not removed by adsorption with cultured bacteria react only with *in vivo* bacteria recovered from the host.

Example 6: Determination of Molecular Weights of Identified Proteins

The molecular weights of proteins identified to be unique or up-regulated under *in vivo* growth conditions were estimated by Whole Band Analysis using the BioImage Computer System (BioImage/Millipore, Ann Arbor, MI). The weights were estimated for the identified bands based on known molecular weights markers. Both Rainbow colored protein molecular weight markers (Amersham Life Science, Arlington Heights, IL) and Bio-Rad SDS-PAGE broad range molecular weight standards (Bio-Rad Laboratories, Hercules, CA) stained with Coomassie blue were used as the standards of comparison.

Example 7: Passive Immunity in Mice

All antibody preparations used in the passive immunity experiments were generated in swine against a primary infection with P. multocida isolate 8261, and in some cases were followed by a second infection with isolate 16926. Serum collected prior to the primary infection was used as a negative control for the passive immunity experiments. Convalescent serum from pig 104 was collected either at 30

had been detergent treated and purified provided intermediate protection. In these groups mice began dying between 6 and 10 days following challenge. No difference in survival time or mortality was seen between the mice that received detergent treated and purified antiserum versus the mice that received antiserum that had been adsorbed with detergent-solubilized culture grown bacteria and then purified. Deaths occurring in these groups suggest that either the total quantity of specific antibody was reduced during purification, or that the half-life of the antibody was shortened by the detergent treatment or purification. In either case, the loss appeared to be non-specific. Western blot analysis of the antibody preparations used in these experiments demonstrated that purification in the absence of adsorption did not remove antibodies that were common to culture grown and *in vivo* grown bacteria and that most reactivity to culture grown bacteria was removed by the adsorption process. Adsorption of the immune serum was performed using isolate 8261. However, Western blot analysis demonstrated that the majority of reactivity against culture-grown isolate 16926 was also removed and that the remaining reactivity against the *in vivo* bands is seen with both the 8261 and 16926 isolates. These observations suggest that the antibodies that remained following adsorption are specific for *in vivo* grown bacterial antigens and that these antibodies are largely responsible for protection against challenge.

Preimmune serum did not afford any passive protection in this model. All mice in this group died between 1 and 3 days post challenge. While the preimmune pig serum reacted with a few bacterial proteins in a Western blot analysis, the reactions were weak (1:10 dilution). This would be expected since the pigs used in these studies were not specific-pathogen free. Although they may have had some exposure to *Pasteurella multocida* or other pathogens with cross-reactive antigens, they were susceptible to

containing 5% aluminum hydroxide gel. Five-fold and twenty-five-fold dilutions of the two vaccines were prepared by diluting the original vaccine in adjuvant. All vaccine preparations were administered by intraperitoneal injection into CF1 mice. Mice were vaccinated twice at a three week interval with a 0.1 ml dose.

All mice were challenged with 50 to 100 CFU of virulent *Pasteurella multocida* isolates 8261 or 16926 and observed for 15 days. As shown in Table 2 below, when the highest concentrations of vaccine were used, both preparations protected mice from both homologous and heterologous challenge. However, when less concentrated vaccines were used, only the vaccine produced from *in vivo* grown bacteria was able to protect the mice against virulent challenge. Eight of ten mice vaccinated with 1×10^7 CFU equivalents of *in vivo* antigens were protected against homologous challenge, and seven of ten mice were protected against heterologous challenge. In contrast, zero of ten mice vaccinated with the least concentrated cultured bacterial antigens survived homologous challenge and only three of ten mice vaccinated with cultured bacterial antigens survived heterologous challenge. None of the ten non-vaccinated mice challenged with isolate 8261 survived, and only one of ten non-vaccinated mice challenged with isolate 19629 survived.

This test of active immunity in mice demonstrated that the addition of antigens that are up-regulated by *in vivo* growth produced a vaccine that was between 5 and 25 times as effective as a vaccine produced from bacteria grown in a standard, enriched media.

secreting antibody specific for the 109 kD protein was cloned twice by limiting dilution and designated as Mab PMA 3-1. The resulting monoclonal antibody was specific for the 109 kD protein produced by *in vivo* grown bacteria and did not react with bacteria grown in complete media.

A second monoclonal antibody was selected based on the ability to bind the 29 kD protein from *in vivo* grown *P. multocida*. Western blot analysis of *in vivo* grown and cultured bacteria demonstrated strong binding to the 29 kD protein of *in vivo* grown bacteria, and very little reactivity to culture grown bacteria. This monoclonal antibody producing cell was cloned by limiting dilution and designated Mab PMA 3-21.

Example 11: Expression of Antigens in Minimal Medium Formulation

Culture conditions were designed to supply *P. multocida* with the minimum level of essential nutrients necessary to support growth, thus mimicking the environment that might be encountered when bacteria invade the host organism. Formulations of minimal medium are shown in Table 3.

TABLE 3

COMPONENT	MEDIUM #1	MEDIUM #2	MEDIUM #3
CARBON SOURCE			
glycerol	40 mM	40 mM	40 mM
sodium lactate	20 mM	20 mM	20 mM
BUFFER			
HEPES	50 mM	50 mM	50 mM
Amino Acids			
L-arginine·HCl0	0.0300%	0.0300%	0.0300%
L-aspartic acid	0.0500%	0.0500%	0.0500%
L-cystine·2HCl	0.0260%	0.0260%	0.0260%
L-cysteine·HCl, anhydrous	0.0790%		

Growth of *P. multocida* in this media resulted in production of the same antigens as produced *in vivo*. Western blot analysis demonstrated that the antigenic profile of bacterial proteins produced in this minimal medium formulation was identical to the antigenic profile of *in vivo* grown bacteria as described in Example 5.

Example 12: Active Protection of Mice by Vaccine Produced from bacteria grown in minimal medium formulation

P. multocida isolate 8261 was cultured in a minimal medium formulation as described in Example 11. Bacteria were also cultured in a standard, enriched media as described in Example 1 or harvested directly from the pleural cavities of infected swine as described in Example 1. All preparations were inactivated with 0.3% formalin with constant stirring for 24 hours at 37° C. The inactivated bacteria were diluted to a pre-inactivation cell count of 1 x 10^9 CFU/ml. The bacterial suspensions were adjuvanted in a squalene emulsion containing Quil A and TDM. Mice (female CF1, Charles River Laboratories) were vaccinated with a 0.1 ml dose by the intraperitoneal route in the lower right quadrant of the abdomen. Mice received two vaccinations three weeks apart and were challenged with either 380 LD_{50} (50% lethal dose) of *P. multocida* 8261 or 209 LD_{50} of isolate 16926 two weeks following the second vaccination.

In this experiment, the vaccine prepared from *P. multocida* cultured in a standard, enriched media containing yeast extract was unable to protect mice from either homologous (8261) or heterologous (16926) challenge. Only one of ten mice survived the 8261 challenge, and only five mice survived the 16926 challenge. In contrast, mice immunized with vaccines prepared from *in vivo* grown bacteria or from bacteria cultured in either of the minimal media formulations had much better survival rates. These survival

in PBS to an optical density of approximately 3.0 and frozen at -70 C until analyzed.

Western blot analyses were performed as described in Example 5. Convalescent antiserum collected from a pig that had been experimentally infected with *A. pleuropneumoniae* (Serotype 5) eight weeks previously was used to develop the immunoblot. The most prominent antigenic difference between the *in vivo* or defined media bacteria and the bacteria cultured in complete HP was the presence of additional bands of approximately 60 kD to 65 kD in the *in vivo* and minimal media preparations. Three different serotypes of *Actinobacillus pleuropneumoniae* (serotypes 1, 5 and 7) were grown in defined media and in complete HP media in order to confirm these differences. In each case, several additional bands were present in the defined media preparation that were not present in the HP grown extract. These antigens were also detected in *in vivo* preparation from the respective serotype.

Similar bacterial preparations from serotype 5 were probed with an antiserum that was specific for a transferrin binding protein that is known to be up-regulated by iron chelation and thought to function in acquiring complexed iron during *in vivo* growth (Deneer and Potter, *Infection and Immunity* (1989) 57(3):798-804). A heavy band at approximately 60 kD was detected in the lanes containing *in vivo* bacteria and in lanes containing bacteria grown in defined media, but not in lanes containing the bacteria grown in complete HP media. This finding confirms that the up-regulation of proteins seen during growth of *Actinobacillus pleuropneumoniae* within the host also occurs when these bacteria are cultured in defined media.

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/216,202

(B) FILING DATE: March 22, 1994

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Thr Val Tyr Asn Gln Asp Gly Thr Lys Val Asp Val Asn Gly
5 10 15

Ser Val Arg Leu Leu Leu Lys Gly Glu Lys Asp Pro Arg Arg Asp
20 25 30

Leu Met Met Asn Gly
35

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Amino Acid

What is claimed is:

1. Antigens of the *Pasteurella*, *Actinobacillus* and *Haemophilus* species of bacteria capable of being up-regulated during infection in a host animal and in minimal medium formulations which provide protection against infections caused by these species.

2. Antigens of claim 1 comprising *Pasteurella multocida* antigens.

3. A *Pasteurella multocida* antigen of claim 2 having a molecular weight, as determined by gel electrophoresis, of approximately 115 kilodaltons.

4. A *Pasteurella multocida* antigen of claim 2 having a molecular weight, as determined by gel electrophoresis, of approximately 109 kilodaltons.

5. A *Pasteurella multocida* antigen of claim 2 having a molecular weight, as determined by gel electrophoresis, of approximately 96 kilodaltons.

6. A *Pasteurella multocida* antigen of claim 2 having a molecular weight, as determined by gel electrophoresis, of approximately 89 kilodaltons.

approximately 29 kilodaltons and an N'-terminal amino acid sequence comprising SEQ ID NO:3.

14. A *Pasteurella multocida* antigen having a molecular weight, as determined by gel electrophoresis, of approximately 34 kilodaltons and an N'-terminal amino acid sequence comprising SEQ ID NO: 1.

15. *Pasteurellaceae* antigens of claim 1 comprising *Actinobacillus pleuropneumoniae* antigens.

16. A vaccine for prevention of an infection by the *Pasteurella*, *Actinobacillus* and *Haemophilus* species of bacteria comprising antigens of *Pasteurella*, *Actinobacillus* and *Haemophilus* species of bacteria which are capable of being up-regulated during infection in a host animal and in a minimal medium formulation.

17. The vaccine of claim 16 wherein the antigens comprise *Pasteurella multocida* antigens.

18. The vaccine of claim 17 wherein at least one *Pasteurella multocida* antigen has a molecular weight of approximately 29 kilodaltons and an N'-terminal amino acid sequence comprising SEQ ID NO:2.

23. The method of claim 22 wherein the Pasteurellaceae infection comprises a Pasteurella multocida infection and the animal is administered an effective amount of a vaccine comprising Pasteurella multocida antigens.

24. The method of claim 23 wherein at least one Pasteurella multocida antigen of the vaccine has a molecular weight of approximately 29 kilodaltons and a N'-terminal amino acid sequence comprising SEQ ID NO: 2.

25. The method of claim 23 wherein at least one Pasteurella multocida antigen of the vaccine has a molecular weight of approximately 29 kilodaltons and a N'-terminal amino acid sequence comprising SEQ ID NO: 3.

26. The method of claim 23 wherein at least one Pasteurella multocida antigen of the vaccine is selected from a group consisting of antigens having molecular weights of approximately 115, 109, 96, 89, 79, 62, 56, 53, and 45 kilodaltons.

27. A method of immunizing healthy animals against infections caused by Pasteurella multocida comprising administering to a healthy animal an effective amount of a vaccine comprising antigens of Pasteurella multocida, wherein at least one Pasteurella multocida antigen of the vaccine has a molecular weight of approximately 34

FIG. 1A

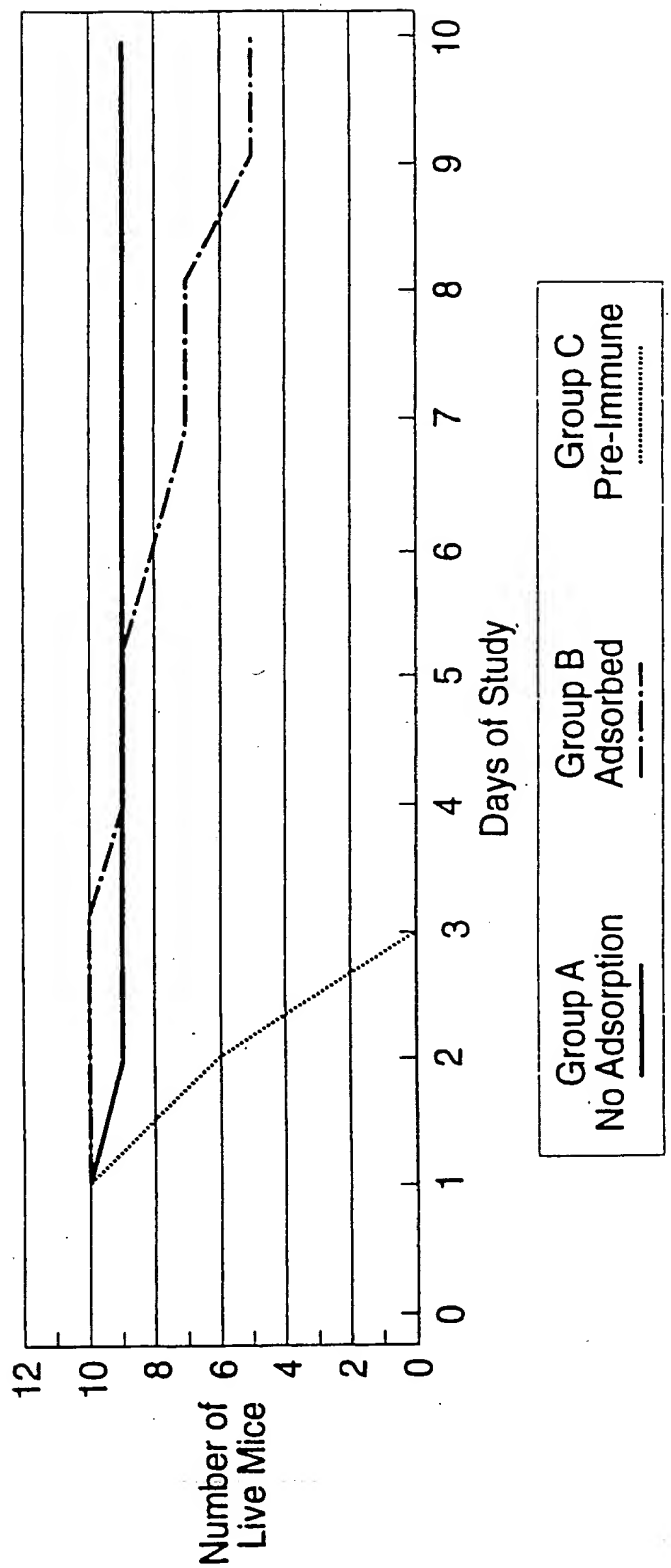

Number of Live Mice
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
9
10
Days of Study
Group A No Adsorption
Group B Adsorbed
Group C Pre-Immune

FIG. 1B

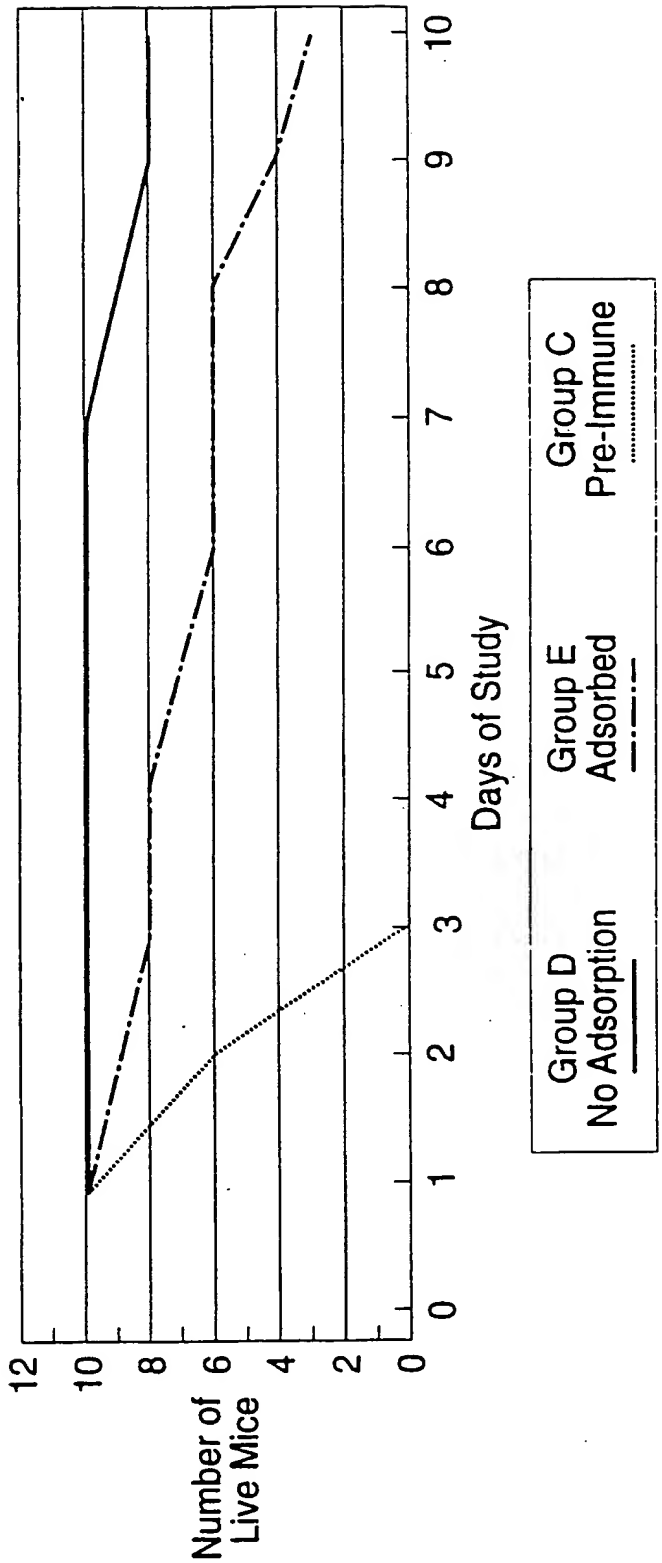
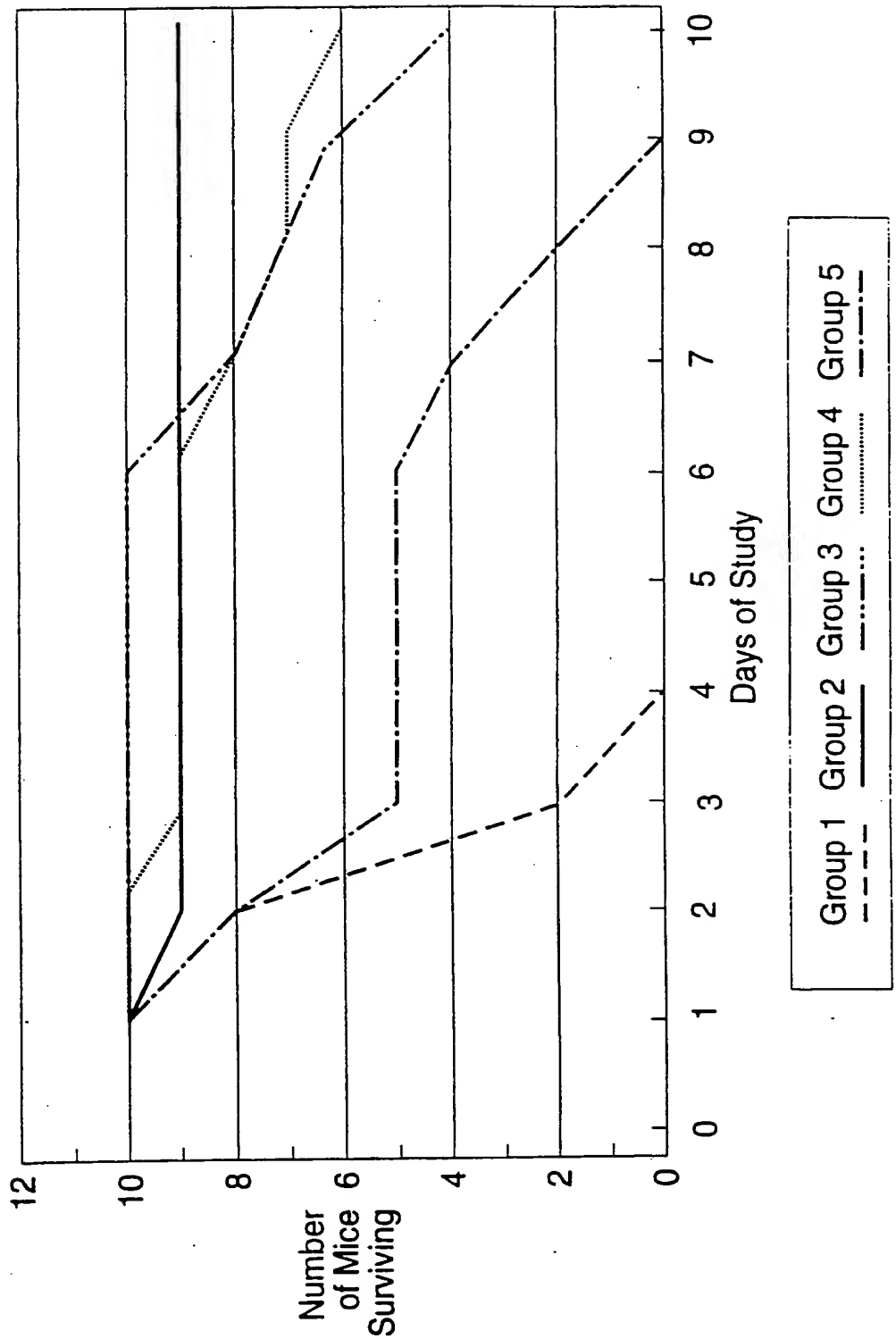

Number of Live Mice
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
9
10
Days of Study
Group D No Adsorption
Group E Adsorbed
Group C Pre-Immune

FIG. 2


Number of Mice Surviving
0
2
4
6
8
10
12
Days of Study
0
1
2
3
4
5
6
7
8
9
10
Group 1
Group 2
Group 3
Group 4
Group 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 95/00185

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/245 A61K39/102

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INFECTION AND IMMUNITY, vol. 61, no. 1, WASHINGTON US, pages 91-96, TAGAWA Y. ET AL. 'Purification and Partial Characterization of the Major Outer Membrane Protein of Haemophilus somnus' see the whole document ---	1-28
A	WO-A-91 15237 (UNIVERSITY OF SASKATCHEWAN) 17 October 1991 see the whole document ---	1-29
A	WO-A-92 11023 (SMITH-KLINE BEECHAM CORPORATION) 9 July 1992 see the whole document ---	1-29
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
25 September 1995	25.09.1995

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)